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Evaluation of a Commercial Enzyme-Based Serum Cholesterol Test Kit for Analysis of Phytosterol and Phytostanol Products

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Plant sterols (phytosterols) have been shown to possess serum cholesterol-lowering properties. In recent years, several phytosterol-enriched functional food products have been developed and marketed. Some phytosterol products contain common unsaturated sterols and some contain a subset of phytosterols called phytostanols (saturated sterols, also called plant stanols). Current methods for the quantitative analysis of plant sterols are labor intensive and require sophisticated gas or liquid chromatographs. In this study, a popular commercial spectrophotometric serum cholesterol test kit was evaluated for the analysis of plant sterols. The results indicate that the method could be modified to analyze phytosterols and phytostanols by increasing the incubation time. Both free phytosterols and fatty acyl phytosteryl esters were quantitatively analyzed, but ferulate phytosteryl esters, such as those that are found in corn and other cereals, were not hydrolyzed by the enzymes in the test kit and therefore were not detected.

KEYWORDS: Phytosterol; phytostanol; analysis; spectrophotometric; cholesterol; enzymatic; nutraceutical

INTRODUCTION

The analysis of cholesterol in human serum and plasma is commonly carried out with a reagent kit that contains three enzymes:

(i) *Cholesterol esterase* (microbial) catalyzes the hydrolysis of cholesterol esters to cholesterol and free fatty acids.

(ii) *Cholesterol oxidase* (microbial) catalyzes the oxidation of cholesterol to cholest-4-en-3-one and hydrogen peroxide.

(iii) *Peroxidase* (horseradish) catalyzes the conversion of 2 hydrogen peroxides + 1 hydroxybenzoic acid + 4-aminoantipyrine to quinoneimine dye (red color, Abs max at 500 nm).

The analysis of plant sterols (phytosterols, including a subset of saturated analogues called phytostanols) is routinely conducted via methods that include gas chromatography (*1*) and HPLC (*2*). The current study was undertaken to attempt to develop a simpler method for phytosterol analysis, one that could be conducted in a laboratory that only possesses basic analytical instruments such as a visible wavelength spectrophotometer or a colorimeter. With growing interest in phytosterols as functional food components, simpler methods for quality assurance/control of phytosterol ingredients and finished products are needed. The present method was developed with this goal in mind.

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MATERIALS AND METHODS

Cholesterol, cholesterol oleate, stigmasterol, stigmastanol, and cholesta-3-one were purchased from Sigma-Aldrich (St. Louis, MO). Oryzanol was obtained from CTC Organics (Atlanta, GA). INFINITY cholesterol reagent was purchased from Sigma-Aldrich or Thermo Electron Corporation (Waltham, MA). Benecol (the label reported that it contained 1500 mg of plant stanol, as fatty acyl ester, per 14 g serving) and Take Control (the label reported that it contained 1650 mg of plant sterol, as fatty acyl esters, per 14 g serving) margarines were purchased from a local grocer. Cholesterol Success (the label reported that it contained 900 mg of free plant sterols per tablet) was purchased from a local vitamin and supplement shop. Cook Smart cooking oil (the label reported that it contained 1120 of mg plant sterol, as fatty acyl ester, per 14 g serving) was obtained on-line from Procter and Gamble when it was test-marketed in 2001. Corn fiber oil was obtained as previously described (*2*). Stigmastanyl-ferulate (also called *trans*-feruloyl beta sitostanol) was synthesized by the method of Condo et al. (*3*).

Stocks of each pure sterol were prepared at a concentration of 0.005 M in 2-propanol. Stocks of each commercial product were prepared to be approximately 0.005 M sterol calculated from product content information (Benecol, Take Control, Cholesterol Success, and Cook Smart). The sterol content for corn fiber oil was estimated to be approximately the same as that of Cook Smart.

For spectrophotometric assays, 50 μl of 0.005 M sterol stock solutions in 2-propanol were added to 5 mL of INFINITY Reagent in a 55 mL tube and the mixture was incubated at 37 °C in a shaking water bath (~60 rpm) for up to 60 min. For spectrophotometric reading, approximately 1 mL of mixture was removed and the Abs at 500 nm was immediately read with a Shimadzu UV-1601 PC UV−Visible dual beam spectrophotometer with pure INFINITY reagent as a reference.

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Table 1. Qualitative Analysis of the Phytosterol/Phytostanol Substrates, Intermediates and Products Created during Incubation of Various Sterols and Stanols with Enzyme Mixtures[a]

substrate	incubation time (min)	steryl/stanyl fatty acyl ester	steryl/stanyl ferulate ester	free sterol	free stanol	ketone 1 (rt = 9 min)	ketone 2 (rt = 3 min)	Abs 500
cholesterol	0	0	0	100	0	0	0	0.000
	5							0.289
	10	0	0	0	0	98	2	0.307
cholesterol:oleate	0	100	0	0	0	0	0	0.000
	5							0.305
	10	0	0	0	0	99	1	0.311
stigmasterol	0	0	0	100	0	0	0	0.000
	5	0	0	30	0	68	2	0.129
	15							0.193
	30							0.227
	60	0	0	0	0	99	1	0.258
stigmastanol	0	0	0	0	100	0	0	0.000
	5	0	0	0	17	2	80	0.176
	15							0.285
	30							0.316
	60	0	0	0	1	2	97	0.319
Take Control	0	98	0	2	0	0	0	0.000
	5	0	0	12	0	86	2	0.298
	15							0.339
	30							0.357
	60	0	0	0	0	97	3	0.357
Benecol	0	99	0	0	1	0	0	0.000
	5	0	0	0	16	0	84	0.315
	15							0.334
	30							0.340
	60	0	0	0	4	0	96	0.383
Cook Smart	0	98	0	2	0	0	0	0.000
	5	0	0	30	0	66	4	0.307
	15							0.389
	30							0.461
	60	0	0	1	0	97	2	0.541
Cholesterol Success	0	0	0	100	0	0	0	0.000
	5	0	0	27	0	58	15	0.230
	15							0.310
	30							0.337
	60	0	0	1	0	80	18	0.350
corn fiber oil	0	55	41	4	0	0	0	0.000
	5	0	39	3	0	47	11	0.186
	15							0.215
	30							0.245
	60	0	36	1	0	50	13	0.275
stigmastanyl-ferulate	0							0.000
	60							0.009
oryzanol	0							0.000
	60							0.006

[a] Blank lines indicate that only Abs was measured.

For HPLC analysis of reactants and products, the mixtures were incubated as above and at the appropriate incubation times, the reaction was stopped by adding 15 mL of 2-propanol, 20 mL of hexane, and 15 mL of water. After gentle shaking of the sample for 5 min and an additional 5 min for phase separation, the top phase was removed, dried under N_2, weighed, and redissolved in 1 mL of hexane with 0.01% BHT.

HPLC analysis of the nonpolar lipids was performed in a binary gradient system as previously described (*2*) with detection via an evaporative light scattering detector.

HPLC-MS of reactants and products was performed using a reversed phase column (LiChrosorb RP-18), with an isocratic mobile phase of 100% methanol, and an Agilent 1100 MSD mass spectrometer operated with atmospheric pressure chemical ionization in the positive mode at a vaporizer temperature of 350 °C, and a capillary voltage of 4000. Under these conditions, the retention times were stigmasterol, 5.8 min; stigmaster-3-one, 4.9 min; stigmastanol, 7.3 min; and stigmastan-3-one, 6.9 min.

All experiments were conducted in triplicate, and the HPLC and spectrophotometric data reported are mean values.

RESULTS AND DISCUSSION

According to the instructions included in the kit, the INFINITY cholesterol reagent is designed to measure free and esterified cholesterol in human serum and plasma samples. Using the recommended incubation of 5 min at 37 °C, we confirmed that both free cholesterol and cholesterol oleate were completely oxidized (**Table 1**). In contrast, phytosterols (stigmasterol) and phytostanols (stigmastanol) were only partially oxidized at 5 min, and required a 60 min incubation for complete oxidation (**Table 1**). Incubation of five phytosterol-containing products for up to 60 min also revealed that this longer time of incubation was required for complete oxidation.

HPLC-ELSD analysis of reactants and products of the reaction of stigmasterol and stigmastanol revealed partial reaction at 5 min and complete reaction at 60 min (**Figure 1**). Sitosterol and the other Δ-5 plant sterols coelute with stigmasterol in this HPLC system. The putative ketone product was the only visible peak at 60 min. The retention time of the ketone

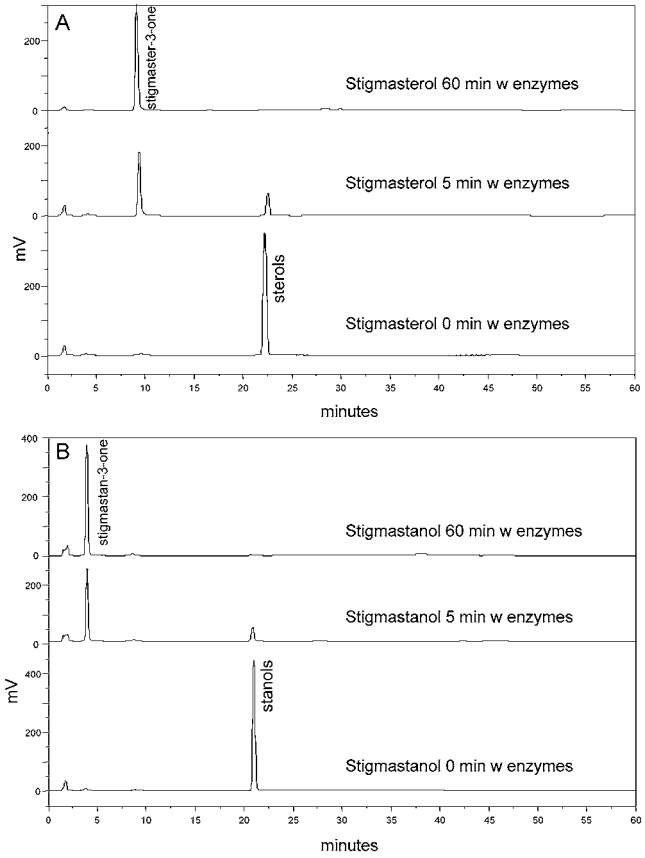

Figure 1. HPLC-ELSD chromatogram of reactants and products from the incubation of (A) stigmasterol and (B) stigmastanol with INFINITY reagent for 0, 5, and 60 min.

product for stigmasterol was about 9 min, and was nearly identical to the retention time of the cholesterol oxidation product, choles-4-en-3-one. The retention time of the ketone product for stigmastanol was about 3.5 min.

LC-MS of stigmasterol (MW 412.7) and the ketone product from its oxidation stigmaster-3-one (MW 410.7) confirmed its expected structure (**Figure 2**). LC-MS of stigmastanol (MW 416.7) and the ketone product of its oxidation, stigmastan-3-one (MW 414.7), confirmed its expected structure (**Figure 3**). It was previously noted (*4*) that with positive APCI, the major ion for sterols is the M − water + 1, and the mass spectra for stigmasterol (**Figure 2a**) and stigmastanol (Figure 3a) verify that this is the major ion for each. The major ions for the two ketone products are both "M + 1," verifying the putative reaction products and indicating that these structures did not lose water during ionization (**Figures 2b** and **3b**). During the oxidation of cholesterol and Δ-5 phytosterols by cholesterol oxidase, the Δ-5 double bond is shifted to Δ-4, making it conjugated to the ketone double bond (*5*, *6*); however, the absence of the Δ-5 double bond (or any other double bonds) in the phytostanols does not seem to have much effect on their rate of oxidation by cholesterol oxidase.

HPLC analysis of the ketone products from a phytosteryl-ester-containing product (Take Control) revealed that the product from this reaction also had a retention time of 9 min (**Figure 4**). Interestingly, all of the triacylglycerols (retention time 3−4 in) in Take Control, Benecol, and Cook Smart, were hydrolyzed by the enzymes in the INFINITY reagent. A large peak of free fatty acids was produced during the reaction of Take Control with the INFINITY reagent (FFA were derived from both steryl esters and triacylglycerols). Because this fatty acid peak had

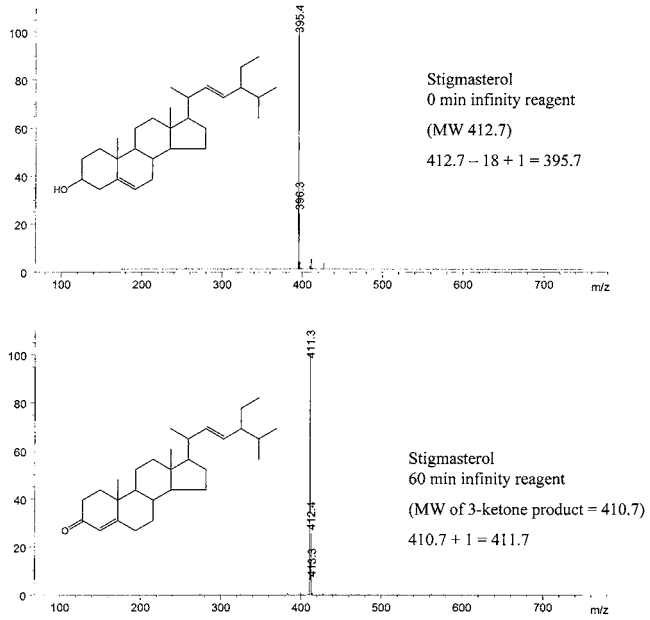

Figure 2. LC-MS-APCI+ mass spectra of stigmasterol and its ketone product produced during incubation with the INFINITY Reagent.

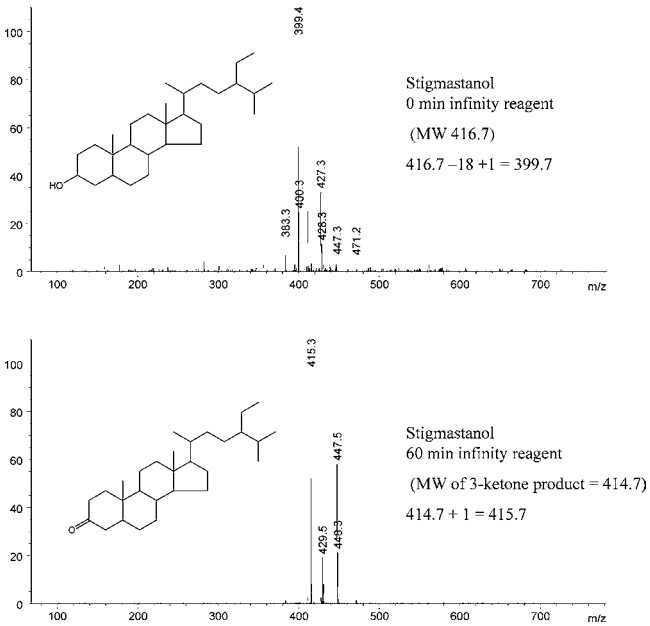

Figure 3. LC-MS-APCI+ mass spectra of stigmastanol and its ketone product produced during incubation with the INFINITY Reagent.

an identical retention time to the ketone product, the extract was treated with diazomethane to convert them to fatty acid methyl esters (retention time 2 min). After diazomethane treatment, a single peak remained at the 9.5 min and was assumed to be the ketone product. HPLC analysis of the ketone product from a phytostanol-containing product (Benecol) revealed that the ketone product from this reaction had a retention time of 3.5 min. Diazomethane treatment was also performed to methylate the fatty acids in the Benecol reaction, but because the retention time of its ketone product was less (3.5 min), the presence of free fatty acids did not mask its appearance.

Unlike the other phytosterol products, corn fiber oil contains a second type of phytosteryl ester, ferulate phytosteryl esters, at a concentration of 3−6 wt % (*2*). HPLC analysis of the products of corn fiber oil + INFINITY reagent revealed that like the commercial products, the phytosteryl fatty acyl esters

Table 2. Estimation of Phytosterol Concentrations in Commercial Products Using the INFINITY Cholesterol Reagent Method, Modified to Include a 60-min Incubation

product	advertised mg of sterol (ester, free) per serving	calculated mmol of sterol per serving	INFINITY values mmol of sterol per serving
Benecol	1500[d]	2.20[a]	2.701 ± 0.012 (23% higher)
Take Control	1650[d]	2.43[b]	2.751 ± 0.010 (13% higher)
Cook Smart	1120[d]	1.65[b]	2.000 ± 0.022 (21% higher)
Cholesterol Success	900[e]	2.17[c]	2.255 ± 0.002 (2% higher)

[a] Calculated assuming the fatty acyl phytostanyl esters are stigmastanol-oleate (MW 681.2). [b] Calculated assuming the fatty acyl phytosteryl esters are sitosterol-oleate (MW 679.2). [c] Calculated assuming that the free phytosterols are sitosterol (MW 414.7). [d] Product has esterified phytosterols. [e] Product has free (unesterified) phytosterols.

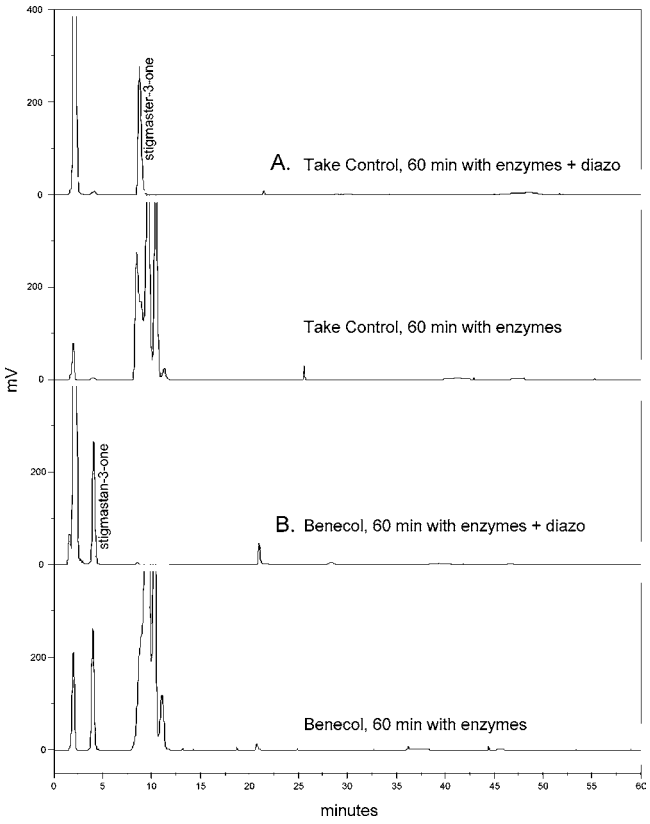

Figure 4. HPLC-ELSD chromatogram of reactants and products from the incubation of (A) Take Control and (B) Benecol with INFINITY reagent for 60 min. Diazomethane was added to convert free fatty acids (rt = 9–11 min) to fatty acid methyl esters (rt = 2 min) and enable the visualization of the stigmasterol ketone product at rt = 9 min.

were completely hydrolyzed (by cholesterol hydrolase), but unfortunately, the ferulate phytosterol esters were NOT hydrolyzed (data not shown). Therefore, when using the INFINITY reagent to analyze phytosterols in corn fiber oil (or other products that contain ferulate phytosterol esters), it appears that the measured value of total sterols will only include free and fatty acyl esters and not ferulate esters. To verify this hypothesis, standards of stigmastanyl-ferulate and oryzanol (a ferulate ester from rice that is comprised primarily of cycloartenyl-ferulate) were then tested separately (**Table 1**). Since both standards only exhibited a trace increase in Abs 500, these data confirm that INFINITY reagent is not capable of detecting ferulate-phytosterol esters.

Finally, the accuracy of this modified assay was evaluated using the four phytosterol/phytostanol products (**Table 2**). Cholesterol was used to construct a standard curve (linear, with a maximum absorbance 500 nm of 0.6 for 0.10 μmol of cholesterol/mL of INFINITY reagent), which was then used to correlate Abs 500 and mmol of sterol per serving. Using this method, the levels of phytosterols in all four products were estimated to be 2−23% higher than indicated by the manufacturers.

Previously, the K_m and V_{max} values for sitosterol and stigmasterol by microbial cholesterol oxidase have been reported (*5*, *7*), but this is the first report comparing the relative reaction rates of phytosterols and phytostanols. These previous references noted that the K_m and V_{max} of 5-α-cholestan-3 β-ol were very similar to those for cholesterol. It is interesting that in the current study stigmastanol (and Benecol) was oxidized at a faster rate than stigmasterol (and Take Control) (**Table 1**). The cholesterol oxidase used in the INFINITY reagent is listed "microbial", and it is possible that the substrate specificity may differ from that of cholesterol oxidases from other species (*5*).

In conclusion, the results of this study indicate that enzyme-based cholesterol test kits may be useful for the analysis of structurally related phytosterols. Most laboratories have access to spectrophotometers or colorimeters. Also, battery-powered miniature units are available for field use, so in theory, this type of assay could be conducted almost anywhere. The products tested in this report contained relatively high concentrations of phytosterols and phytostanols (>10%), and this spectrophotometric assay was sufficiently sensitive. To measure endogenous levels of phytosterols in fruits and vegetables, it may also be possible to use enzyme-based methods, but it will probably be necessary to use fluorometric assays since they have higher sensitivity than spectrophotometric assays. Several fluorometric cholesterol-oxidase-based kits are commercially, but their use would require access to a fluorometer.

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